

Stem Cells and Early Lineage Development

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DOI 10.1016/j.cell.2008.01.039

The recent derivation of pluripotent stem cell lines from a number of different sources, including reprogrammed adult somatic cells, raises the issue of the developmental equivalence of these different pluripotent states. At least two different states representing the epiblast progenitors in the blastocyst and the pluripotent progenitors of the later gastrulating embryo have been recognized. Understanding the initial developmental status of the different pluripotent lines is critical for defining starting conditions for differentiation toward therapeutically relevant cell types.

Pluripotent embryonic stem (ES) cells are unique tools for studying mammalian development and differentiation in the Petri dish (see Review by C.E. Murry and G. Keller in this issue of *Cell*). When first derived from mouse blastocysts, they were thought to represent a self-renewing “frozen in time” version of the inner cell mass (ICM) of the blastocyst. This is because ICM cells, like ES cells, are capable of making all somatic cell types of the mouse, including the germline, when returned to the blastocyst environment. However, in such chimeras, mouse ES cells do not contribute to the extraembryonic lineages of trophoblast and primitive endoderm, suggesting that they are pluripotent and not totipotent. This restriction in potency is identical to that of the epiblast progenitors in the ICM (Chazaud et al., 2006), suggesting that ES cells may represent an in vitro model of early epiblast development. More recently, pluripotent stem cell lines with properties similar to mouse ES cells have been derived from a variety of developmental stages and mammalian species, as well as from adult cells reprogrammed by ectopic transcription factors (Table 1). However, the relationships of all of these cell lines to the lineages established in the early embryo are not clear. By comparing the ability of these cell lines to differentiate into extraembryonic lineages and to the early germ layers of the embryo, I propose that there are at least two different pluripotent states represented among the currently available pluripotent stem cell lines and that, paradoxically, the cell lines representing the epiblast progenitors of the blastocyst may be more restricted in their

developmental potential than those representing the pluripotent progenitors of the later-stage gastrulating embryo.

Stem Cells from the Mouse Blastocyst

By the time of implantation, the mammalian blastocyst has developed three distinct cell lineages. An outer layer of trophectoderm encloses the blastocoelic cavity, at one end of which is the ICM. The ICM consists of epiblast or primitive ectoderm, covered by a monolayer of primitive endoderm on the blastocoelic surface (see Figure 1). Extensive lineage tracing and chimera studies in the mouse have shown that these lineages are restricted in fate by the time of implantation, with the trophectoderm and primitive endoderm giving rise to extraembryonic cell types of the placenta and yolk sacs and the epiblast giving rise to the entire fetus (Yamanaka et al., 2006). This restriction in lineage of the primary cell types is mimicked by the behavior of the three different self-renewing stable progenitor cell lines that can be derived

from the mouse blastocyst, namely ES cells, trophoblast stem (TS) cells, and extraembryonic endoderm (XEN) cells (Yamanaka et al., 2006). The undifferentiated ES cell state is dependent on the expression of a combination of transcription factors, most notably Oct4, Sox2, and Nanog, and by signaling through the cytokine leukemia inhibitory factor (LIF) and bone morphogenetic protein 4 (BMP4) (Ying et al., 2003). TS cells are derived from the trophectoderm, require expression of Cdx2, Eomes, and other TS-specific transcription factors, and depend on FGF and activin/nodal signaling for self-renewal. XEN cells show morphological and molecular similarities to primitive endoderm derivatives, express lineage-specific transcription factors, such as GATA-6 and Sox7, and require exogenous FGF signaling for their derivation, but not necessarily for ongoing maintenance. All three cell lines recapitulate the lineage of their appropriate blastocyst precursor when injected into blastocysts to generate chimeras (Yamanaka et al., 2006).

Table 1. Properties of Different Pluripotent ES Cell-like Cells

Cell Line	In Vitro Pluripotency	Spontaneous Trophoblast Differentiation	Chimera Formation	Growth Factors for Self-Renewal
mES	+	–	+	LIF, BMP
mEG	+	?	+	FGF/SCF, then LIF
mGS	+	?	+	GDNF, then LIF
mEpiSC	+	+	–	FGF, activin
miPS	+	?	+	LIF
hES	+	+	na	FGF, activin
hEG	+	?	na	FGF
hEpiSC	+	?	na	FGF

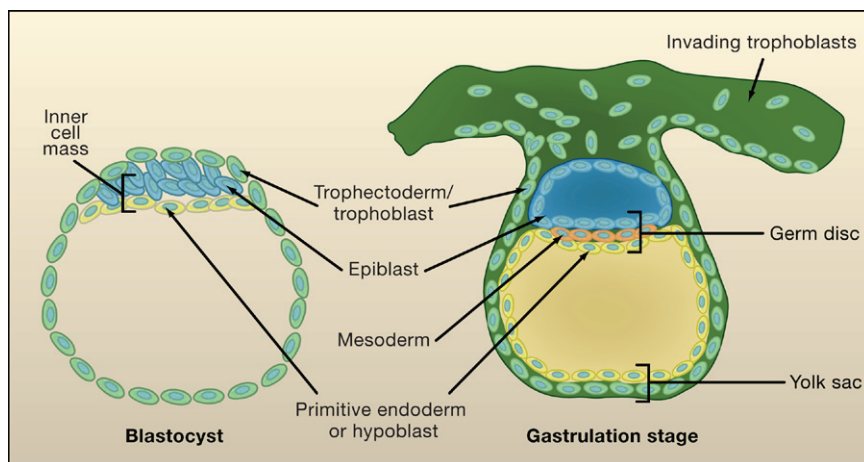


Figure 1. Implantation of the Human Blastocyst

Shown is the human blastocyst prior to implantation (left) and the human conceptus shortly after implantation as gastrulation begins (right). The inner cell mass (ICM) of the blastocyst forms the epiblast of the germ disc, which is underlain by the primitive endoderm. As gastrulation begins, the primitive streak in the middle of the disc generates a mesoderm layer that separates the epiblast and primitive endoderm. Definitive endoderm will also be generated to replace the primitive endoderm. The trophoblast of the blastocyst produces invasive syncytiotrophoblasts that promote the attachment and invasion of the conceptus into the uterine wall.

Interestingly, FGF signaling acting through the canonical Ras-MAPKinase pathway seems to play opposing roles in ES cells versus TS cells, promoting differentiation in ES cells and proliferation in TS cells. ES cells lacking FGF4 or Erk2 are resistant to differentiation and maintain expression of pluripotent markers, suggesting that there is an autocrine action of FGF in preparing ES cells for differentiation (Kunath et al., 2007; see Essay by J. Silva and A. Smith in this issue). Conversely, TS cells require exogenous FGF signaling through the ERK pathway for maintenance of their self-renewal; blocking the FGF signaling pathway leads to their differentiation into trophoblast giant cells (Rossant, 2001). These different roles for FGF signaling reflect the *in vivo* roles of FGF signaling in early development. FGF signaling is required to maintain proliferation of the early postimplantation trophoblast, as well as to promote differentiation of the epiblast. Although ES cells do not readily generate trophoblast under normal culture conditions, they can be transformed into functional TS cells by conditional deletion of the *Pou5f1* gene (which encodes the Oct4 protein) or by overexpression of Cdx2 (a caudal-related homeodomain transcription factor) and transfer to TS culture conditions (Niwa, 2007).

Based on the lineage restriction of mouse ES, TS, and XEN cells in chimeras and other properties, we have proposed that these cell lines represent the three restricted lineages known to exist by implantation in the mouse blastocyst. Implicit in this proposal is that manipulating the differentiation of ES cells into later cell types will require mimicking the phases of early axis formation, followed by germ layer development, before specialized cell types can be obtained.

Do Human Blastocyst-Derived Stem Cells Obey the Same Rules?

Primate blastocysts prior to implantation are very similar in structure to rodent blastocysts, although cell number is higher and postimplantation morphology is different. Although the morphology of the cell lineages of the primate blastocyst is similar to that of the rodent, there could be differences in timing of lineage restriction, given the longer period of time before implantation (7–10 days versus 4 days in mouse). When ES cells were first derived from human blastocysts, they were shown to possess many of the properties found in mouse ES cells. These properties include expression of Oct4, Sox2, and Nanog and pluripotency (demonstrated *in vitro* and when the human ES cells were transplanted to ectopic sites in mouse

where they formed teratomas) (Thomson et al., 1998). However, the genome-wide transcriptional profiles of human and mouse ES cells show many differences, and there is limited overlap in the downstream targets of Oct 4 and Sox2 as defined by genome-wide ChIP analysis (Boyer et al., 2005; Loh et al., 2006). The significance of these differences should not be overstated as the studies were performed on different technology platforms and under different culture conditions. However, they are intriguing, especially when combined with evidence that the signaling pathways supporting self-renewal may also differ. LIF signaling does not support self-renewal of human ES cells, whereas BMP actively promotes their differentiation. A combination of FGF and activin/nodal signaling maintains self-renewal of human ES cells under serum-free conditions (Vallier et al., 2005). These are exactly the conditions that promote TS cell not ES cell development from the mouse blastocyst and drive differentiation of mouse ES cells (Kunath et al., 2007). Human ES cell cultures have been reported to contain cells with trophoblast-like expression patterns (Thomson et al., 1998, Xu et al., 2002). However, stable proliferating TS cell lines have not yet been reported either directly from human blastocysts or from ES-cell derived trophoblast cells. The reason may lie in the behavior of human trophoblast cells around the time of implantation, where there is rapid formation of a nonproliferating syncytiotrophoblast that mediates attachment and invasion of the uterine epithelium. Only at later stages does the trophoblast show extensive proliferation to form the chorionic villi. Thus, the blastocyst may not be the appropriate stage to obtain FGF-responsive proliferating TS cells.

Human ES Cells and EpiSCs: Defining a Common Epiblast Cell State

Clearly, there are distinct differences between mouse and human ES cells, despite their common properties of Oct4 expression, ease of self-renewal, and pluripotency. On the face of it, the simplest explanation would be that human ES cells are in a pre-epiblast stage of commitment, where all lineage pathways including trophoblast are still open, whereas mouse ES cells represent the

already lineage-restricted epiblast progenitor of the blastocyst. This difference could relate to the slower pace of lineage restriction in the early stages of human development compared with mouse. However, recent work has suggested a different explanation. Two groups derived pluripotent cell lines, termed EpiSCs, directly from the early postimplantation epiblast in the mouse (Brons et al., 2007; Tesar et al., 2007). These cell lines still depend on the standard pluripotency transcription factors, Oct4, Sox2, and Nanog, but proliferate in the presence of FGF and activin rather than LIF. EpiSCs were able to generate tissues from all three germ layers *in vitro* and to form teratomas. However, they were unable to contribute to normal tissues in mouse chimeras. Whether this is due to a true developmental block or a cellular incompatibility between EpiSCs and the host embryo, such that the EpiSCs fail to integrate into the host ICM, is currently unclear. As well as requiring the same growth factors for self-renewal as human ES cells, EpiSCs were also reported to show gene expression profiles and transcription factor networks closer to human ES cells than to mouse ES cells. Interestingly, like human ES cells, EpiSCs could express markers of trophoblast and primitive endoderm when treated with BMP4 (Brons et al., 2007).

The similarities between human ES cells and mouse EpiSCs have led to the suggestion that human ES cells are actually equivalent to the early postimplantation epiblast, rather than its ICM progenitor. Data mining of the deposited gene expression arrays for EpiSCs shows that they do express pluripotent markers but also seem to be expressing higher levels of mesoderm and definitive endoderm transcripts as compared to mouse ES cells (B. Cox and J.R., unpublished data). This suggests that EpiSCs, and by extension human ES cells, may be in an unusual transition state that is perhaps more equivalent to a primitive streak progenitor: poised on the verge of differentiation into multiple directions. Consistent with this possibility, FGF and activin play roles in both proliferation and differentiation of the postimplantation epiblast *in vivo*. However, primitive streak cells would be expected to be even less likely to differentiate into extra-

embryonic lineages, the trophoblast and primitive endoderm, than the earlier epiblast progenitors of the blastocyst. And yet both human ES cells and mouse EpiSCs have been reported to have this property. This paradox is not easily resolved by embryological comparisons and suggests that the process of generating permanent self-renewing cell lines may change the epigenetic environment and open up pathways of differentiation not normally revealed *in vivo*. Because FGF and activin, the factors used to promote human ES cell self-renewal, also promote TS cell self-renewal, any tendency toward trophoblast differentiation in human ES cells or mouse EpiSCs will be accentuated by expansion of these cells. Conversely, in mouse ES cells, FGF treatment drives extensive differentiation, such that formation of any trophoblast cell would probably be missed. In fact, mouse ES cells have been reported to generate some trophoblast differentiation under certain culture conditions (Schenke-Layland et al., 2007).

The Growing List of ES Cell-like Cells

The first pluripotent stem cell lines to be isolated were embryonal carcinoma (EC) cells derived from multilineage tumors known as teratocarcinomas (Solter, 2006). Spontaneous teratocarcinomas can arise in the adult testis or ovary of mice or humans. They can also be produced experimentally by grafting early mouse embryos to ectopic sites. Although EC cells can make chimeras, contributions are often low, germline transmission has never been consistently reported, and chimeras often develop EC-derived tumors. Whether these properties are more similar to ES cells or EpiSCs is not really clear. EC cell lines, like P19, which were derived from ectopic grafts of postimplantation embryos, might be closer to EpiSCs than to mouse ES cells. How much of the variable properties of mouse EC cells relates to possible differences in lineage origin and how much to the known karyotypic anomalies of these cells is unclear.

Importantly, it seems that all the stages of development from which it is possible to derive teratocarcinomas can also give rise to ES cell-like pluripotent cells directly *in vitro*. The first ES cell-like

cells to be derived from nonblastocyst sources were mouse and human EG cells, which are derived from primordial germ cells in the developing gonad (Matsui et al., 1992; Resnick et al., 1992; Shambloott et al., 1998). In the mouse, these cells behave like ES cells in their pluripotent capacity but have imprinting differences that relate to their germ cell origin. Initial cultures of primordial germ cells (PGCs) in both mouse and human require FGF signaling for proliferation, but established EG cultures seem to respond to growth factor signaling in a similar manner to the ES cells of the appropriate species. The epiblast-like properties and the extraembryonic potential of mouse and human EG cells have not been extensively studied, making it unclear whether they show the same potential lineage differences as mouse and human ES cells.

Three groups have reported the derivation of pluripotent ES cell-like cells from the adult or neonatal mouse testis (Guan et al., 2006; Kanatsu-Shinohara et al., 2004; Seandel et al., 2007). After initial expansion of spermatogonial stem cells in the growth factor GDNF, a few colonies resembling ES cells were observed and could be expanded in LIF into permanent cell lines. These multipotent germline stem (mGS) cells, when derived from the neonatal testis, were fully pluripotent as judged by somatic and germline chimerism after blastocyst injection and are assumed to be ES cell-like rather than EpiSC-like. Interestingly, Shinohara and colleagues reported that epiblast-like colonies were also observed in the spermatogonial cultures (Kanatsu-Shinohara et al., 2004), raising the question of whether EpiSCs could also be obtained if cultures were supplemented with FGF and activin.

When considering all of these ES cell-like cells, there is simply not enough data available to assess whether they all fall into the two proposed pluripotent states, ES cell-like and EpiSC-like, or, indeed, whether other pluripotent states exist. It is clear that only cells that express Oct4 and have the potential to form germ cells or are germ cells themselves can give rise to pluripotent cell lines directly. Although germ cells develop as a small group of cells in the posterior of the primitive streak, the entire epiblast up until that time is thought to have germ

cell potential. Once Oct4 is downregulated and germ cells are set aside, the epiblast loses the capacity to make germ cells and to make teratocarcinomas. One would predict that the ability to generate EpiSCs from epiblast would similarly be lost concomitant with germ cell segregation, a testable hypothesis. Whether there is a special epigenetic state associated with pluripotency, perhaps related to this germ cell potency, is a subject of much current research. However, the overriding importance of the key transcription factors, Oct4 and Sox2, for driving pluripotency is supported by the recent derivation of ES cell-like cells, called iPS cells, from mouse (Takahashi and Yamanaka, 2006; Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007) and human fibroblasts (Takahashi et al., 2007; Yu et al., 2007). Reprogramming of the adult cells was achieved by retroviral expression of a set of exogenous transcription factors, which must always include Oct4 and Sox2, as well as factors to promote cell proliferation (see Review by R. Jaenisch and R. Young in this issue). Exactly the same set of transcription factors—Oct4, Sox2, Klf4, and c-Myc—were able to induce the formation of iPS cells from mouse and human adult somatic cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006), but the growth factor conditions required for expansion were clearly different. Human iPS cells could be expanded in FGF but not in the presence of LIF, whereas mouse iPS cells required LIF. Not reported is whether mouse reprogrammed cells can be expanded into an EpiSC-like cell in the presence of FGF and activin.

The Developmental State of ES Cell-like Cells

There is now a growing array of mouse and human cell lines with pluripotent capacity, but their relationship to each other and to their lineage progenitors in the embryo is less than clear. In the mouse, ES cells are well characterized and their full pluripotency can be proven by analysis of chimeras. ES cell-like cells generated from the testis and by reprogramming of adult fibroblasts have also been shown to possess full pluripotency, including germline contribution in chimeras. However, the growth factor conditions used to maintain mouse ES cells

and ES cell-like cells seem to be peculiar to the mouse. It has not proven possible to isolate fully pluripotent ES cells directly from the blastocyst of any other mammalian species, including the rat, even in the presence of LIF. Further, the LIF/JAK/STAT signaling pathway does not appear to be required *in vivo* for normal epiblast development. Instead, it plays a special role in delayed implantation, promoting epiblast survival (Nichols et al., 2001). Exogenous LIF may thus be a very potent inhibitor of the differentiation pathway toward the postimplantation epiblast that would be the normal fate of the epiblast progenitors in the ICM. In species that lack mechanisms to delay implantation, this pathway may not be available for preserving the epiblast progenitor as an ES cell-like cell. On the other hand, the growth factor conditions including FGF and activin that are used to maintain self-renewal of human ES cells and mouse EpiSCs may reflect conserved pathways involved in germ layer development in all vertebrate species. This raises the possibility that EpiSC-like cells may be a more common form of pluripotent progenitor across different species. Indeed, it has been reported that rat EpiSCs can be readily derived under the same conditions as mouse EpiSCs (Brons et al., 2007). It remains to be seen whether non-LIF-mediated conditions can be found that promote the proliferation of cells equivalent to mouse ES cells from other species. Further detailed comparison of the developmental potential of all the different ES cell-like cells in terms of their spontaneous differentiation into extraembryonic cell types, their responses to ectopic expression of extraembryonic lineage-specific transcription factors, and their responses to changing the growth factor environment should help to clarify the developmental equivalence of each cell type.

Knowledge of the initial developmental state of the different pluripotent cell lines is not just a matter of biological curiosity. Responses to differentiation protocols will vary according to where the cells sit in a developmental hierarchy. Protocols developed in mouse ES cells may not be easily transferable to human ES cells if they are nonequivalent in developmental status. It is likely that mouse ES cells have to be differentiated through an epiblast/

primitive streak stage first, before definitive germ layer differentiation, whereas human ES cells may already be at this transitional stage. Mouse EpiSCs and human ES cells, for example, express some markers typical of definitive endoderm and may be easier to drive toward this therapeutically important cell lineage compared with mouse ES cells, which tend toward differentiation into primitive endoderm. The possibility that there is more than one pluripotent ES cell-like state becomes even more important to understand in the context of reprogramming adult cells to pluripotency. Understanding the different pathways that can lead to reprogramming should help to clarify the range of different pluripotent states.

ACKNOWLEDGMENTS

I thank Brian Cox for data mining and Austin Smith, Peter Rugg-Gunn, and Cheryle Seguin for helpful discussions.

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